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Care during freeze-drying of bovine pericardium tissue to be used as a biomaterial: A comparative study ☆

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ABSTRACT

Bovine pericardium (BP) tissue is widely used in the manufacture of bioprosthetics. The effects of freeze-drying on the BP tissue have been studied by some researchers in order to decrease their cytotoxicity due to preservation in formaldehyde solution, and to increase the lifetime of the product in storage. This study was undertaken in order to study the effect of freeze-drying in the structure of BP. To perform this study BP samples were freeze-dried in two different types of freeze-dryers available in our laboratory: a laboratory freeze-dryer, in which it was not possible to control parameters and a pilot freeze-dryer, wherein all parameters during freezing and drying were controlled. After freeze-drying processes, samples were analyzed by SEM, Raman spectroscopy, tensile strength, water uptake tests and TEM. In summary, it has been demonstrated that damages occur in collagen fibers by the loss of bulk water of collagen structure implicating in a drastic decreasing of BP mechanical properties due to its structural alterations. Moreover, it was proven that the collagen fibrils suffered breakage at some points, which can be attributed to the uncontrolled parameters during drying.

Introduction

Biological tissues have been used since the 1960s as alternative biomaterials to the prosthetic mechanical heart [4]. Since 1974, bovine pericardium (BP) has become one of the most commonly used materials for the manufacture of bioprosthetics [7].

BP is an anisotropic material composed mainly of collagen fibers and elastin embedded in an amorphous matrix, which is constituted of proteoglycans and hyaluronic acid. Collagen fibers are arranged in layers, with different alignment directions on each layer, giving rise to interesting mechanical properties of the pericardium, including the ability to undergo large deformation during the execution of physiological functions [10]. It is important to note that BP basically comprises two sheets: the fibrous pericardium (parietal sheet) and by serous pericardium (epicardium or visceral layer). The fibrous pericardium is composed of a loose arrangement of collagenous and elastic fibers (loose connective tissue); while serous pericardium, which faces the epicardium, is composed of mesothelium with its basal lamina overlying a thin layer of loose connective tissue [28]. The advantage of using this tissue is its high content of collagen, in which modifications can be performed in amine (—NH_2), carboxyl (—COOH) and hydroxyl (—OH) groups [27]. To stabilize and crosslink the tissue, BP is usually treated with glutaraldehyde (GA). Crosslinks reduce the biodegradability and antigenicity of the tissue, modify its mechanical properties, and reduce its thrombogenicity [4]. However, GA treatment is toxic and can induce calcification *in vivo* [15,32,2], leading to valve failure and the need of prosthesis replacement [34,11,30].

Several authors have applied freeze-drying technique in biomaterials with the aim of preservation and consequently use them for replacing or restoring organs or damaged tissues, promoting the compatibility of these materials with the physiological environment [14,33,24,13,12,9]. Some authors have also studied the preservation of tissues by cryopreservation [25,8,35]. However, in case of freeze-drying some care must be taken throughout the process in order to preserve structural and functional features of the tissue.

Freeze-drying can be defined as the drying of a given substance through its freezing and subsequent removal of associated solvent with the direct sublimation, without passing through the liquid phase. Usually the solvent is water [6]. Freeze-drying process involves three main steps: freezing, primary drying and secondary drying. After freezing the water is removed from the material by sublimation (primary drying). Subsequently, water that remained unfrozen in the first stage is removed by desorption under reduced pressure. Freezing is considered one of the most important stages of the process. After freezing the structure, size and shape of the product are fixed. Freezing defines the size and distribution of ice crystals in the material, and this has an influence on the characteristics of the primary and secondary drying stages [29,26]. If the

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structure of the matrix is altered during freeze-drying it may suffer damage and even result in loss of the product. The thermal treatment annealing can be applied during the freezing stage to bring greater uniformity of size and distribution of ice crystals in the matrix. In annealing, the product is maintained at a specific freezing temperature (above glass transition – Tg – and below the melting temperature of ice crystals in the material) for a period of time to allow the reorganization of ice crystals in the matrix. Then the temperature is taken below the Tg and maintained so that the material does not collapse during primary drying [16,31,1]. Annealing before freeze-drying [22] could also be useful to facilitate the incorporation of chemical agents into bovine pericardium tissue.

In addition, Maizato et al. 2003 [23] demonstrated that, compared with conventional glutaraldehyde-treated bovine pericardium, freeze-dried pericardium is less cytotoxic, with less residual glutaraldehyde. The work developed by Aimoli et al. 2007 [3] suggests that freeze-drying of bovine pericardium tissue before treatment with chemical substances (crosslinkers) appears to prevent calcification of the matrix.

A comparative study between two common ways to obtain dried biomaterials was conducted. Specimens were freeze-dried in two different freeze-dryers: the laboratory freeze-dryer and the pilot freeze-dryer.

This study was undertaken in order to study the effect of freeze-drying in the structure of biological tissues (bovine pericardium).

Materials and methods

Bovine pericardium preparation

Bovine pericardium was collected at a slaughterhouse, cleaned, washed, and stored in glycerol (89% v/v) for preservation. Before use, BP was washed with saline solution (NaCl 0.9% w/v aq.). Specimens were freeze-dried in two different freeze-dryers: the laboratory freeze-dryer (Group A) and the pilot freeze-dryer (Group B).

Group A: Samples were prepared in the laboratory freeze-dryer in which the freezing step was performed in an ultra freezer at −70 °C for two hours, held in a freezer −20 °C for one hour to anneal treatment, and then the temperature was decreased until −70 °C for two hours). The total time of freeze-drying process was 24 h. The vacuum applied during both primary and secondary drying was 750 mTorr.

Group B: Samples were prepared in the pilot freeze-dryer according to the specifications described by our group [5], using the slow freezing protocol with annealing treatment. Briefly, specimens were frozen at −40 °C for two hours, to anneal treatment the temperature was raised to −20 °C for one hour, and then the temperature was decreased until −40 °C for two hours. Primary drying was carried out at −5 °C and secondary drying at 25 °C (for final time see Fig. 1). The pressure used for both primary and secondary drying was 160 mTorr.

Degree of swelling

Samples (4 cm^2) were weighted and immersed in an excess of water (50 mL). Water uptake was measured in terms of weight increment over the time. The swelling degree was determined by the following equation:

$$St = wt - wi/wi \times 100$$

Where: St is the degree of swelling at time t as a percentage, wt is the final mass in grams and wi is the initial mass in grams. The test was performed in triplicate for each sample.

Raman spectroscopy

Raman analyses were performed in order to determine the second structure of freeze-dried BP membranes. The samples were analyzed in a FT–Raman FRA106/S (Bruker), using 4 cm^{-1} of resolution, a laser set point of 250 mW and 512 scans.

Tensile test

The tensile test was performed in a TA-XT2 Texture Analyzer, (Stable Micro Systems) with cell load of 245.1662 N and sensitivity of 0.009806 N. The test speed was 15 mm/min according to the ASTM D638 test for type V samples. The applied tension was increased until sample failure. Each sample group was subjected to 50 tests. After testing, the data collected were analyzed using the MATLAB program to determine the Young's modulus (E) and rupture tension (σ_{rup}).

Scanning electron microscopy (SEM)

BP samples (1 cm^2) were attached to the SEM support, and sputtered with gold for 5 s. BP micrographs were analyzed and captured using a JSM 7401-F (Jeol). The analysis was performed in duplicate for each sample.

Transmission electron microscopy (TEM)

Specimens (1 cm^2) were fixed in 2% glutaraldehyde (Sigma) for two hours and in cacodylate buffer for 30 min at room temperature. Specimens were further fixed in osmium tetroxide (Sigma), dehydrated in increasingly concentrated grades of alcohol, and embedded in Spürr resin. Ultra-thin sections (70 nm) were stained with uranyl acetate and lead citrate. The observations and photographic records were made in a 906-E transmission electron microscope (LEO) at a voltage of 80 kV (IPEN/USP), using of 50.000-times magnification. The analysis was performed in duplicate for each sample.

Results

Freeze-drying process

Fig. 1 represents the graph generated from the data monitored by the pilot freeze-dryer after freeze-drying process of BP according to the parameters studied by Borgognoni et al. 2009 [5]. This treatment was performed by freezing the product at a temperature of −40 °C for two hours, raising the temperature of the product to −20 °C for one hour, then decreasing the temperature to −40 °C for two more hours. After this step, it is possible to note that the chamber pressure is reduced and the product temperature increased to −5 °C to initiate the drying process. The dew point shows the occurrence of primary drying and secondary drying (after the 1261 min data point), when the temperature of the plate rises to 25 °C, as does the temperature of the product.

Scanning electron microscopy

The samples freeze-dried in the laboratory freeze-dryer (Group A) apparently suffered some fibers breakage in the fibrous pericardium (Fig. 2D), while samples of group B appear to be intact. Observing the serous pericardium (Fig. 2A and B), both samples showed integrity, with no sign of deformity or disruption of the tissue.

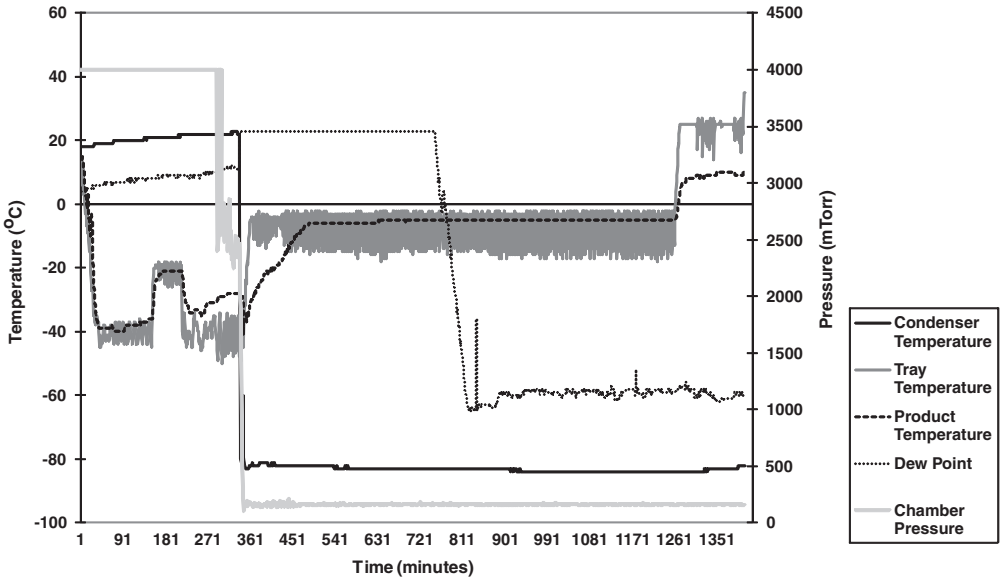

Fig. 1. Freeze-drying chart of bovine pericardium generated by the pilot freeze-dryer.

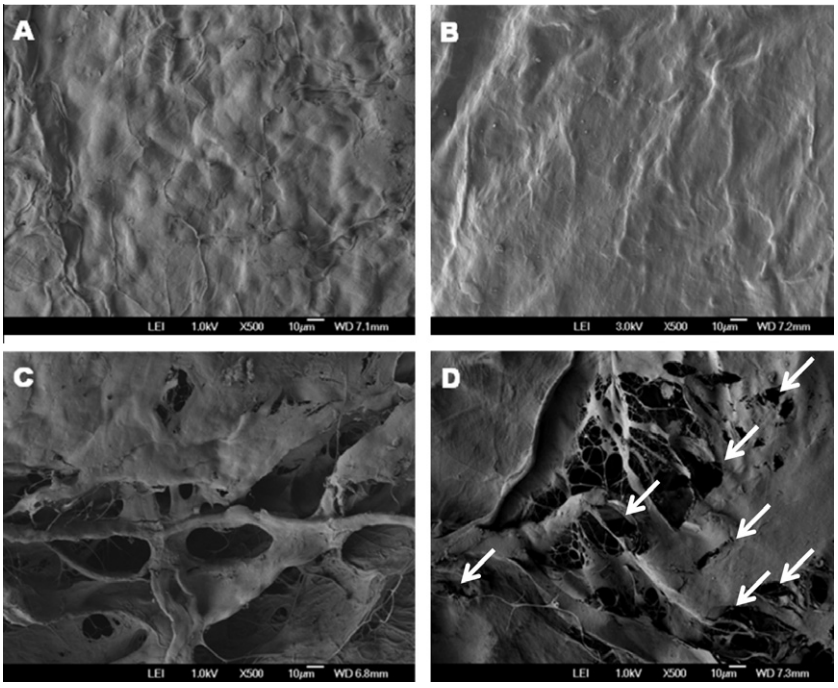

Fig. 2. Micrographs of the serous pericardium (A and B) and fibrous pericardium (C and D) from group B (A and C) and from group A (B and D). White arrows indicate rupture of collagen fibers. All scale bars in 10 μm.

Raman spectroscopy

Raman spectroscopy, showed in Fig. 3, revealed that the characteristic peaks related to the structure of type I collagen (Amide I, Amide III and δ-NH) were maintained in both samples [18,20,22]. However, is possible to note considerable alterations on the peaks intensity for the samples freeze-dried on the laboratory freeze-dryer (Group A). The second derivative method (Savitzky–Golay) was applied to a spectral treatment in order to confirm the difference in the intensities. This treatment makes an adjustment in the base line and smoothing of 21 points. According to the second derivative, the peaks responsible for the collagen triple-helix structure are stronger when freeze-drying was performed in the pilot freeze-dryer (Group B).

Mechanical properties

According to the data generated by MATLAB (Table 1), when BP is freeze-dried by the laboratory freeze-dryer (Group A) Young's Modulus (E) – reflecting the elasticity of the material – is drastically decreased. The E value decreases from 196.53 MPa (Group B) to 108.56 MPa (Group A). Rupture tension (σ_{rup}), which is the maximum stress that a material can withstand, was also negatively

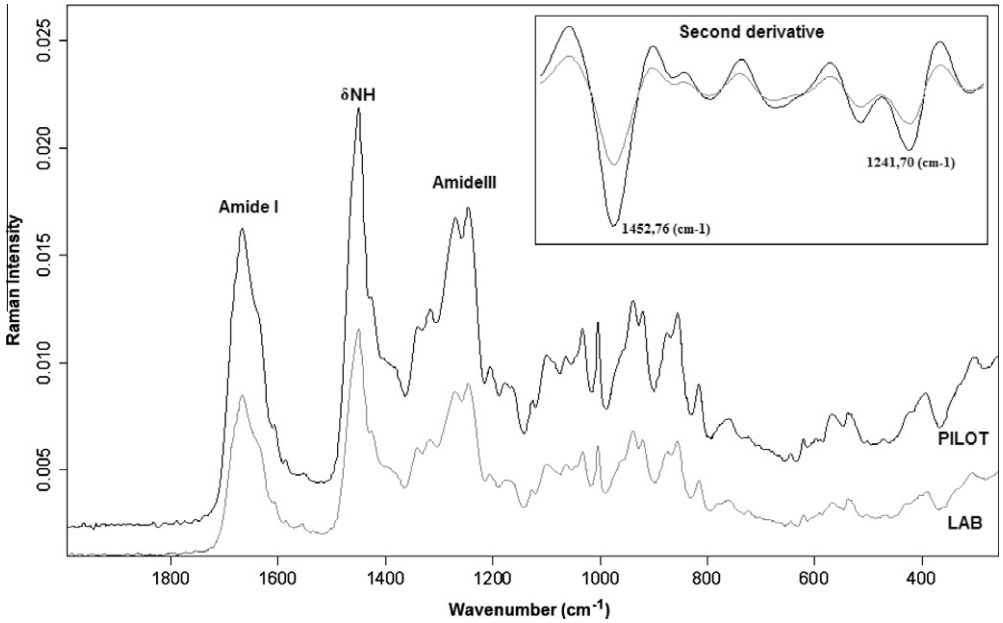

Fig. 3. Raman spectra from freeze-dried BP samples by laboratory (gray) and pilot (black) freeze-dryers.

Table 1
Data obtained by tensile test for samples lyophilized by two different freeze-dryers.

Sample	E* (MPa)	σ_{rup}** (MPa)
Pilot (Group B)	196.53 ± 83.94	18.93 ± 9.61
Laboratory (Group A)	108.56 ± 40.70	12.26 ± 5.20

* E: Young's modulus.
** σ_{rup}: Rupture tension. (n = 50).

affected for group A samples, decreasing from 18.93 MPa (Group B) to 12.26 MPa (Group A).

Swelling kinetics

Fig. 4 shows that samples in group B have a lower degree of swelling when compared with group A. Moreover, it is noticed that water absorption tends to stabilize faster for group B than for group A – after 4 h 30 min of testing compared to 7 h 30 min.

Transmission electron microscopy

In the micrograph (Fig. 5A) it is possible to note points where rupture of collagen fibers occurred along the tissue (black arrows) when BP was freeze-dried by the laboratory freeze-dryer (Group A). On the other hand, TEM analysis for group B showed that the tissue was better preserved, since most of collagen fibers appeared unbroken (Fig. 5B).

Discussion

BP is composed mainly of type I collagen. The tropocollagen triple helix structure is stabilized by the interchain hydrogen bond formation. Parallel tropocollagen molecules are covalently cross-link with each other through their aldehyde and amino groups, forming collagen fibrils. Collagen self-organizes to form bundles or a meshwork that determines the tensile strength, the elasticity and the geometry of the tissue [17]. The loss of structural water of this protein can cause alterations on both structural and conformational state.

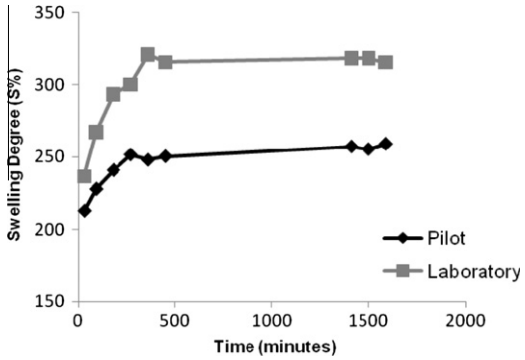

Fig. 4. Swelling kinetics of samples freeze-dried by two different freeze-dryers. Gray = group A; Black = group B.

To perform this study, bovine pericardium samples were freeze-dried in two different types of freeze-dryers available in our laboratory: a laboratory freeze-dryer (Group A) and a pilot freeze-dryer (Group B). In a laboratory freeze-dryer the freezing stage was done in a separate ultra freezer (samples were placed at −70 °C ultra freezer for two hours, to anneal treatment the samples were maintained in a freezer for one hour at −20 °C; finally, samples were placed at −70 °C ultra freezer for two more hours). In addition, during freeze-drying it was not possible to control parameters such as pressure (the whole process was performed at a pressure of 750 mTorr), shelf and sample temperature, and humidity. A pilot freeze-dryer allows the whole process to be controlled by the operator. From the chart (Fig. 1) it is possible to observe the tray temperature, product temperature, condenser temperature, primary drying and secondary drying (dew point) and the chamber pressure, which are crucial parameters during freeze-drying. The dew point, which is monitored by a hygrometer inside the drying chamber, indicates the amount of moisture in the air. The higher the dew point, the higher the moisture content at a given temperature. As can be seen in the graph, a thermal treatment (annealing) was performed during the freezing step.

After freeze-drying processes, samples were analyzed by SEM, Raman spectroscopy, tensile strength, water uptake tests and

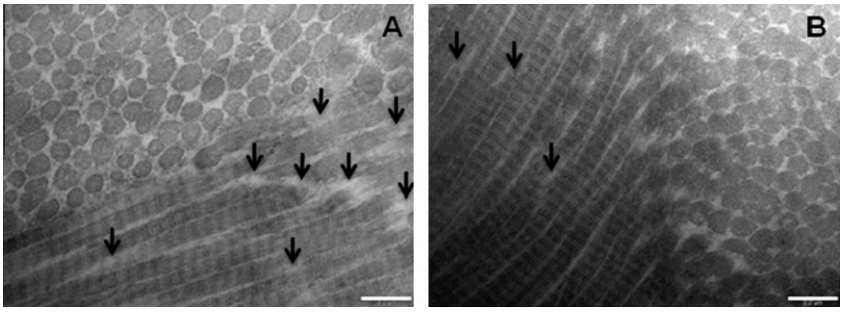

Fig. 5. TEM micrographs of lyophilized BP. A = group A; B = group B. Black arrows indicate rupture of collagen fibers. All scale bars 0.2 μm.

TEM, in order to evaluate the types of structural changes undergone by the tissue, and how they can affect the mechanical properties of tissue.

The micrographs obtained by SEM (Fig. 2) shows that the superficial structure of the tissue after freeze-drying depends greatly on drying conditions. It is possible to note on Fig. 2D that the membrane suffered alterations on the fibrous pericardium that appear to be disruptions of collagen fibers. These modifications occurred mainly in the fibrous side probably due to the loose arrangement of collagen and elastic fibers when compared to serous pericardium [28]. Furthermore, the lost of this arrangement can be occurring by the loss of structural water from the tropocollagen triple helix during the drying stage. This assumption had been confirmed by the Raman spectroscopy results.

Raman spectroscopy is a powerful technique used to evaluate the chemical structure and the conformation arrangement of molecules. To understand the impact of both freeze-drying processes on the water removal from a protein it is important to analyze its secondary structure and correlate it with the drying process [1]. Raman spectra of the group A and group B samples demonstrated that the fingerprints peaks for type I collagen (Amide I and Amide III) are presented in both samples. The main difference of the spectra collected for both samples is the intensity of these peaks. The intensity peaks for group A samples is lower than group B samples. Amide III band (1240–1250 cm^{-1}) related to C—C stretch form α-helical type I collagen showed alterations on the peak intensity. The same behavior was noticed to the Amide I peak (~1665 cm^{-1}), which is attributed to C=O stretching [18]. Besides, at 1004 cm^{-1}, the intensity of this peak was considerable lower for group A samples. This peak is related to the loss of bulk water from collagen structure [21]. The loss of bulk water on collagen leads to a great difference in structural state of BP tissue, which modified the tissue leading to a reduction of both the elasticity and rupture tension of the material, as discussed below.

The traction test allows the identification of mechanical properties of the BP tissue samples (Table 1). For example, the Young's modulus decreased 44.76% when samples were freeze-dried by the laboratory freeze-dryer. Besides, rupture tension reduced 35.24% for samples from group A. Based on the results we can infer that the modifications suffered by BP, with major effects in the fibrous pericardium, led to a drastic decrease in mechanical properties when freeze-drying was performed in the laboratory freeze-dryer. The loss of bulk water left the tissue more susceptible to breakage.

Water uptake test was applied in order to evaluate the membrane properties for their possible use as a biomaterial. The ability of a membrane to rehydrate quickly and preserve water is an important aspect especially in case of application of this tissue as a heart valve substitute, which needs to execute the best performance as a bioprosthesis. The water uptake test (Fig. 4) revealed that swelling degree for group A samples is superior then group B samples. This result indicates that the modifications occurred on BP membranes leave the tissue looser with more space between collagen fibers.

TEM analysis is used to successfully obtain structural information of type I collagen [19]. TEM micrographs showed that in fact collagen fibril suffered breakage at some points (black arrows). This behaviour occurs mainly when freeze-drying was performed by the laboratory freeze-dryer in a ratio of 8:3 when compared to the pilot freeze-dryer (Fig. 5).

In summary, it was proven that freeze-drying of bovine pericardium tissue should be performed with controlled parameters to ensure the integrity of collagen fibers, and consequently leading to a better performance in bioprosthesis. Moreover, in this work it has been demonstrated that damages occur in collagen fibers by the loss of structural water of tropocollagen triple helix implicating in a drastic decreasing of BP mechanical properties due to its structural alterations. We can expect that this work has pointed out that freeze-drying of other biological tissues should be carefully studied to determine the appropriate freeze-drying parameters to a better preservation of the biomaterial structure.

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